Changes in the concentrations of steroids and prostaglandin F in preovulatory follicles of the mare after administration of hCG

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Summary. Fluid was aspirated from the preovulatory follicle of Group 1 mares ($N = 6$) when follicles reached 32–34 mm in diameter. Group 2 mares each received an i.v. injection of hCG when the preovulatory follicle reached 35 mm. Aspiration of follicular fluid was performed 28–32 h after treatment. Follicular fluid was aspirated from Group 3 mares 28–32 h after the preovulatory follicle reached 35 mm in diameter.

Concentrations of progesterone were significantly higher in follicular fluid from Group 2 mares than in that from mares in Groups 1 and 3. Testosterone was significantly higher in follicular fluid from Groups 2 and 3 than in Group 1 mares. There were no significant differences among groups in concentrations of oestradiol and prostaglandin F (PGF) in follicular fluid.

Keywords: oestradiol; progesterone; testosterone; PGF; follicle; mare

Introduction

In many species there is extensive information on concentrations of steroids and prostaglandins (PGs) in follicular fluid in the preovulatory period. In the mare, however, investigations have been limited to collection of follicular fluid from mares slaughtered on certain days of the oestrous cycle or classification of follicular status retrospectively by histological examination.

At the beginning of oestrus, oestradiol is the dominant steroid secreted by the preovulatory follicle of the mare (Kenney *et al.*, 1979; Meinecke *et al.*, 1987). The oestradiol is probably largely synthesized from testosterone which is the main steroidal product of the theca interna from preovulatory follicles (Tucker *et al.*, 1986). Fay & Douglas (1987) reported that concentrations of testosterone in fluid collected from the presumptive preovulatory follicle after ovariectomy significantly increased between Days 1 and 4 of oestrus, whereas concentrations of oestradiol and progesterone did not change; however, because events were timed from the first day of behavioural oestrus, it was not possible to relate hormone concentrations to ovulation.

In other species, concentrations of PGs increase shortly before ovulation and this increase is thought to be mandatory for ovulation (Armstrong *et al.*, 1974; Killick & Elstein, 1987; Munalulu *et al.*, 1987). In mares, elevated concentrations of PGF have been measured in fluid from follicles showing histological evidence of atresia (Kenney *et al.*, 1979) but no serial samples have been collected from preovulatory follicles. Indirect evidence exists, however, for the involvement of PGs in ovulation in the mare. Fenprostalene, a synthetic analogue of PGF with an extended half-life of 24 h, hastened follicular maturation and time of ovulation in mares injected during oestrus (Savage & Liptrap, 1987).

In mares the preovulatory follicle may be identified by ultrasound examination *per rectum* when it is approximately 32 mm in diameter (Pierson & Ginther, 1985). Ovulation occurs at a mean

diameter of 45 mm, 6 or more days later (Pierson & Ginther, 1985). Administration of hCG to mares when the preovulatory follicle is 35 mm in diameter results in ovulation 36–48 h later (Duchamp *et al.*, 1987). Therefore, by using timed follicular puncture after hCG administration, the maturity of the follicle may be assured. In the present study, fluid was aspirated from the preovulatory follicle of oestrous mares by a colpotomy procedure during early follicular growth (32–34 mm), or 28–32 h after the follicle was 35 mm in diameter in mares receiving or not receiving an i.v. injection of hCG. Concentrations of progesterone, oestradiol-17 β , testosterone and PGF were measured in the follicular fluid.

Materials and Methods

Animals and follicular aspiration

Animals. Light horse mares, 2–15 years of age, were teased daily with a stallion to determine onset of oestrus. When mares were in oestrus, ovaries were examined daily *per rectum* by palpation and ultrasound to monitor follicular development and measurements of follicular diameter were made using ultrasound. On the first day of oestrus the 18 mares were assigned randomly to one of 3 groups. Fluid was aspirated from the preovulatory follicles of Group 1 mares ($N = 6$) on the day that follicles first reached 32–34 mm in diameter. Group 2 mares ($N = 6$) each received an intravenous injection of 2000 i.u. hCG (Butler Co., Columbus, OH, U.S.A.) on the day the preovulatory follicle was first seen to be 35 mm or greater in diameter. Aspiration of follicular fluid was performed between 28 and 32 h after injection. The preovulatory follicles from Group 3 mares ($N = 6$) were aspirated between 28 and 32 h after the examination at which follicular diameter was first discovered to be 35 mm or greater in diameter.

Follicular aspiration. A colpotomy procedure was used to aspirate follicular fluid (Hinrichs & Kenney, 1987). Mares were sedated by administration of 100 mg xylazine (Bayvet Division Miles Laboratories Inc., Shawnee, KS, U.S.A.) and 20 mg acepromazine (TechAmerica Group Inc., Elwood, KS, U.S.A.) i.v., with 10–15 mg butorphanol (Bristol Laboratories Division of Bristol-Meyers Co., Syracuse, NY, U.S.A.) i.v. as an analgesic. The perineum was scrubbed with a povidone–iodine solution and the operator's hand was introduced into the peritoneal cavity via an incision in the cranial vagina. A trocar was inserted into the peritoneal cavity through the abdominal musculature of the flank. The ovary was manipulated by the hand in the abdomen and the follicular fluid aspirated via a needle inserted through the trocar.

Radioimmunoassays

Oestradiol-17 β . The method used has been described by Watson *et al.* (1987). The antiserum (5322) cross-reacted with oestradiol-17 β 100%, oestriol 0.6%, oestrone 0.4% and other steroids tested <0.1%. Recovery of radiolabelled oestradiol following a double extraction of 1:10 000 dilution of follicular fluid in ethyl ether was 92%. The within- and between-assay coefficients of variation were 11 and 17% respectively. The assay blank was lower than the lowest standard and sensitivity with reference to the standard curve was 2.5 pg/ml. Accuracy determinations yielded a linear regression of $y = 1.33 + 0.93x$ and a correlation coefficient of 0.99.

Progesterone. Progesterone was measured using a kit (Diagnostic Products Corporation, Los Angeles, CA, U.S.A.) with ^{125}I -labelled progesterone as a tracer. The assay was modified to include standards (2.5–1000 pg) prepared in assay buffer rather than in the human plasma provided. Samples were diluted 1:20, 1:50 and 1:100 and extracted twice with petroleum ether. The antiserum had the following cross-reactivities: progesterone 100%, 11-deoxycorticosterone 1.7%, 11-deoxycortisol 2.4%, 20 α -dihydroprogesterone 2%, 5 β -pregnan-3,20-dione 1.3% and other steroids tested <1%. The limit of sensitivity of the assay was 25 pg/ml. The within- and between-assay coefficients of variation were 6 and 9% respectively.

Testosterone. Follicular fluid (0.1 ml) was extracted twice in double-distilled water (1 ml) with methyl-*t*-butyl ether (5 ml) and assayed at 1:10 dilution. Standards were prepared in methanol and ranged from 10 to 500 pg/0.1 ml. [1,2,6,7- ^3H]Testosterone (0.1 ml; 10 000 cpm; Amersham, Arlington Heights, IL, U.S.A.) was added both to samples and standards, followed by antiserum (Wein Laboratories, Inc., Succasunna, NJ, U.S.A.; lyophilized plasma reconstituted to 35 ml; 0.1 ml per tube). The assay was incubated at 4°C for 2 h. After addition of 0.5 ml charcoal–dextran (0.25% charcoal; 0.025% dextran), samples were incubated for 10 min at 4°C. The tubes were then centrifuged at 2000 g for 10 min at 4°C and the supernatant poured into scintillation vials. Scintillation fluid was added (3.5 ml; 4 l toluene:15 g PPO) and the vials counted in a scintillation counter after overnight equilibration. The antiserum had the following cross-reactivities: 5 α -dihydrotestosterone 60%, Δ^1 -testosterone 49%, 5 α -androstan-3 α ,17 β -diol 3%, Δ^5 -androsten-3 β ,17 β -diol 16%, 5 α -androstan-3,17-dione 2%, aldosterone 5%, androstenedione 2% and other steroids tested <1%. The assay blank was non-detectable and sensitivity of the assay with reference to the standard curve was 50 pg/ml. Within- and between-assay coefficients of variation were 7 and 10%, respectively. Accuracy determinations yielded a linear regression of $6.08 + 0.70x$ and a correlation coefficient of 0.99.

PGF. Prostaglandin F was measured directly in follicular fluid and at a 1:5 dilution. The assay method has been described by Watson *et al.* (1987). Standard curves prepared in assay buffer and in follicular fluid showed good parallelism. Their coefficient of correlation was 0.92. Sensitivity of the curve was 0.25 ng/ml. Cross-reactivities of the antiserum (>0.1%) were with 100% PGF-2 α , 75% with PGF-1 β , and 1.5% with PGF-2 β . The within-assay coefficient of variation was 4%. All samples were included in one assay.

Statistical analysis

Concentrations of hormones in different treatment groups were compared using a one-way analysis of variance. To stabilise the variance, data were log₁₀ transformed. Values of $P < 0.05$ were considered to be significantly different.

Results

Table 1 shows concentrations of steroids measured in follicular fluid from the three groups of mares. Concentrations of progesterone were significantly higher in follicular fluid from Group 2 than from Group 1 ($P < 0.001$) and Group 3 ($P < 0.02$) mares but no significant difference existed between Group 1 and Group 3 mares. Testosterone concentrations were significantly higher in follicular fluid from Group 2 than from Group 1 ($P < 0.01$) mares. Fluid from Group 3 mares contained significantly higher testosterone concentrations ($P < 0.05$) than did that from Group 1 mares. Concentrations of oestradiol in follicular fluid were not significantly different between groups. Concentrations of PGF from all of the Group 1 mares and from all but one of the Group 3 mares were below the detection limit of the assay. No significant differences existed between groups in concentrations of PGF.

Table 1. Concentrations (ng/ml) of progesterone, testosterone, oestradiol and PGF in follicular fluid aspirated from mares in Groups 1, 2 and 3

	Group 1 (N = 6)	Group 2 (N = 6)	Group 3 (N = 6)
Progesterone	20.2 $\pm$ 8.82 ^a	401.5 $\pm$ 146.6 ^b	98.3 $\pm$ 83.62 ^a
Testosterone	5.9 $\pm$ 1.02 ^a	12.1 $\pm$ 1.49 ^b	15.0 $\pm$ 4.45 ^b
Oestradiol-17 β	894 $\pm$ 166.8	1059 $\pm$ 248.4	1160 $\pm$ 232.7
PGF	<0.3	18.8 $\pm$ 10.84	0.7 $\pm$ 0.37

Values are mean $\pm$ s.e.m.

Means with different superscripts are significantly different ($P < 0.05$) when the data are log₁₀ transformed.

Discussion

The present study shows that, at 28–32 h after treatment with hCG, intrafollicular concentrations of progesterone, testosterone and probably PGF increase. Previous studies have shown that between 82 and 89% of mares with a follicle of 35 mm in diameter and treated with hCG ovulate within 48 h, and most of these will ovulate between 24 and 48 h (Loy & Hughes, 1966; Sullivan *et al.*, 1973; Duchamp *et al.*, 1987). By contrast, only 38% of mares which had a follicle of 34 mm at the start of the study (similar to Group 3 mares), ovulated by 48 h (Duchamp *et al.*, 1987). In the present study, therefore, ovulation was imminent in the hCG-treated mares.

In the mare, previous data showed a tendency for larger preovulatory follicles to contain higher concentrations of progesterone than smaller, less mature follicles (Fay & Douglas, 1987). There is

also histological evidence of luteinization as the follicle matures towards ovulation (Kenney *et al.*, 1979). The present study demonstrated approximately a 20-fold increase in progesterone concentrations as ovulation approached, which is similar in magnitude to the increase reported for pre-ovulatory rat follicles (Munalulu *et al.*, 1987). In mammals, species differences apparently exist for requirement of progesterone for ovulation. In the ewe, progesterone is an intermediary in the action of LH in the mechanism of ovulation (Murdoch *et al.*, 1986) and treatment with antiserum against progesterone decreases ovulation rates in rats (Mori *et al.*, 1977). Progesterone does not appear to be necessary, however, for the ovulatory process in rabbit ovaries perfused *in vitro* (Holmes *et al.*, 1985).

In the mare, as in other species, there is evidence that testosterone is produced by the theca interna under the control of LH (Tucker *et al.*, 1986). The increase in testosterone measured in Group 2 and 3 mares in the present study can therefore probably be attributed to the high circulating concentrations of LH during oestrus, and the large increase in thecal LH receptors in the preovulatory follicle during late oestrus (Fay & Douglas, 1987). Similar results have been reported for other species (Dieleman *et al.*, 1983; Munalulu *et al.*, 1987).

The large increase in the oestrogen-synthesizing ability of the horse follicle occurs when the follicle reaches about 30 mm in diameter (Kenney *et al.*, 1979), which is earlier than the period of study in the present investigation. This study showed that, in the mare, oestradiol concentration in follicular fluid did not increase in the period immediately before ovulation. In women (Lumsden *et al.*, 1986), sheep (Hay & Moor, 1975) and cattle (Dieleman *et al.*, 1983) there is a drop in concentrations of oestradiol in the preovulatory follicle, which may be due to the inhibitory effects of LH on oestradiol synthesis observed *in vitro* (Moor, 1974). The absence of a measurable decrease in oestradiol in horse follicles before ovulation may be due to the difference in the preovulatory LH profile. In the mare, the preovulatory LH surge is relatively prolonged and peak concentrations are not attained until after the time of ovulation (Alexander & Irvine, 1982) whereas, in other species, ovulation occurs several hours after peak plasma LH concentrations.

The results of this study showed that, in the mare, concentrations of PGF tended to increase in follicular fluid before ovulation. It is probable that this pattern reflects gonadotrophin stimulation of PG production from follicular cells (Patwardham & Lanthier, 1981). In other species, rising concentrations of PGs in follicular fluid appear to be necessary for successful ovulation (Armstrong *et al.*, 1974; Murdoch *et al.*, 1986; Killick & Elstein, 1987) by mediating changes in vasculature and collagen degradation and synthesis in the follicle wall (Murdoch & Myers, 1983; Okamura *et al.*, 1985). The requirement for PGs in the ovulatory process in the mare has not been established.

It will now be necessary to investigate periovulatory hormonal changes in horse follicles in more detail, in association with administration of pharmacological substances directly into the preovulatory follicle to determine factors controlling ovulation.

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